SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF SURFACE MODIFIED PAMAM DENDRIMER WITH GALLIC ACID FOR ANTI-PROLIFERATIVE EFFECTS IN CANCER CELLS

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ABSTRACT

Cancer is the second leading cause of death worldwide. Over the past decade, several strategies have been applied to reduce the toxicity and deleterious side effects of anti-cancer drugs. In clinical practices frequently used chemotherapeutic anti-cancer drugs are plant derivatives, but they have adverse cytotoxic effects and limited efficacy for drug resistance. Many natural derivatives have broad spectrum biological and anti-cancer activities, but due to limited bioavailability can't use at clinical level. Gallic acid (GA) is a natural polyphenol, which exhibits a broad spectrum of biological activities, but its therapeutic application was limited due to poor bioavailability. Since recent past to overcome the obstacle of anti-cancer therapy significant attention has been drawn to improve drug delivery system. The present study was aimed to improve drug delivery method using nano carrier PAMAM dendrimers. In the present study GA was conjugated with 4.0 G PAMAM dendrimer at outer surface. The conjugation of GA with PAMAM dendrimers has been characterized by biophysical methods and further examined its bioavailability, anti-cancer activity and molecular mechanism of cell death in in vitro and in vivo model system of cancer. The results show that PAMAM-GA conjugate inhibits cell proliferation of different origin of cancer cells and induces mitochondria-dependent apoptosis in HCT-116 cells. PAMAM-GA conjugate showed less cytotoxic response as compared to the free Gallic acid to the normal cells. The results show that PAMAM-GA conjugate inhibits cell proliferation of different origin of cancer cells, improves cellular uptake of GA, inhibits colonogenic ability, restricts cancer cell migration by down regulating the expression of MMP-9, inhibits NF-kB activation and release of pro-inflammatory cytokines to manifest apoptotic cell death in HCT-116 cells rather than necrosis. Interestingly, in vivo study shows that PAMAM-GA conjugate restricts tumor growth in DL lymphoma xenograft mouse model. Moreover, anti proliferative effects PAMAM-GA conjugate was examined in drug resistant HCT-116 cells. The results show that PAMAM-GA conjugate induces apoptotic cell death as well as Rhodamine accumulation in HCT-116/R cells. PAMAM-GA conjugates downregulate the expression of P-gp that responsible for drug resistant in cancer cells. In conclusion findings of this study revealed that PAMAM-GA conjugate improve the bioavailability of GA specificity towards cancer cells and manifests apoptotic cell death in HCT-116 and HCT-116/R cells. This indispensable approach may be beneficial for the revolution of anticancer therapy.

1. Introduction

1. Introduction

Cancer is a life-threatening disease that can affect anyone regardless of age, lifestyle, gender or ethnicity. Prognosis of cancer has improved greatly in modern times owing to treatment advances and early detection programs but still treatment of cancer is challenging (Chatterjee, Chattopadhyay *et al.* 2016). Most frequent therapy including surgery, radiotherapy and chemotherapy used against cancer. The dynamic characteristics of cancer and tumor growth create difficulty in treating and delivering drugs only to the specific tissue type. Current cancer treatments include surgical intervention, radiation and chemotherapeutic drugs, which have accomplishes adverse response to the patient side effects and after some time of treatment develops drug resistance. The development of drug resistance causes major obstacle in successful application of cancer therapy. Cancer cells become drug resistant in many ways such as cell death inhibition, alteration of drug target, inactivation of drug, efflux of the drug, Epithelial Mesenchymal Transition (EMT), DNA damage repair (Zahreddine and Borden 2013). Multidrug resistance of cancer cells is a potentially barrier to effective chemotherapy of cancer. ATP-binding cassette (ABC) transporters, including MDR1 (ABCB1), MRP1 (ABCC1) and ABCG2, can confer multidrug resistance to cancer cells. MRP2 (ABCC2), MRP3 (ABCC3), MRP4 (ABCC4), MRP5 (ABCC5), ABCA2 and BSEP (ABCB11) are responsible to transporting drugs and have an imperative role in drug resistance (Gottesman, Fojo *et al.* 2002). The major mechanism of multidrug resistance in cancer cells is the energy dependent drug efflux pump known as the P-glycoprotein (P-gp) or multi drug transporter (Ueda, Cardarelli *et al.* 1987). In the 1970s, P-gp was found as one of the biomarkers for the classical multidrug resistance (MDR) (Juliano and Ling 1976). The efflux pump, P-gp is the product of *MDR*1 gene in human (Chen, Chin *et al.* 1986) and the product of two different related genes, *mdr*1*a* and *mdr*1*b* in the mouse (Croop, Raymond *et al.* 1989). There are 48 ABC transporters present in the human (Chen, Chin *et al.* 1986). Many chemotherapeutic drugs are derivatives of natural polyphenols or flavonoids. The major obstacles include their toxicity or poor bioavailability. Since past decades several natural plants derived such as vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, etoposide, derived from epipodophyllotoxin, and paclitaxel has been identified as anti-cancer drugs (da Rocha, Lopes *et al.* 2001). Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids,

alkaloids, and flavonoids, which have been found in vitro to have anticancer properties. Gallic acid, Quercetin, Glucosides, Catechins, free Hydroxycinnamic acids and Anthocyanins, which are absorbed in the small intestine and highly excreted in bile that showed great variability in the bioavailability of polyphenol (Manach, Williamson *et al.* 2005).

Gallic acid (3,4,5-trihydroxy benzoic acid) is a natural polyhydroxy phenolic compound, generally found in various plants, fruits and foods which is responsible for its anti-cancer activity (Kahkeshani, Farzaei *et al.* 2019). In recent past, GA has been shown chemopreventive and anti-proliferative activities against a variety of human cancers (Verma, Singh *et al.* 2013). GA is known to have has a broad spectrum of biological activities including antioxidant, anti-inflammatory, anti-microbial anti-viral anti-allergic anti-melanogenic and anti-mutagenic activities etc. (Badhani, Sharma *et al.* 2015). Similar to other natural polyphenolic derivatives, GA also has poor bioavailability, limited tissue distribution and rapid degradation, which limit its clinical application to consider as drug candidate (Manach, Williamson *et al.* 2005, Brglez Mojzer, Knez Hrncic *et al.* 2016).

Cancer treatment is very much dependent on the method of delivery. The effectiveness of a cancer therapy device is measured by its ability to reduce and eliminate tumors without damaging healthy tissue (Moding, Kastan *et al.* 2013). Therefore, a distinct capacity to target tumors is essential in the success of the therapeutic device. Targeted drug delivery to cancer cells or tumor vasculature is a striking approach to fight against cancer. Recent advances in nanotechnology have shown a greater interest in drug delivery system. In past decades several molecules have been identified with excellent biological properties but could not be used as a drug due to poor bioavailability including limited solubility, systemic toxicity, renal clearance, metabolic degradation and impermeability in crossing the blood brain and intestinal barriers (Wen, Jung *et al.* 2015). Therefore, using nano carriers could be best approach to accomplish specific delivery of a drug at the desire site, for desire time and at desire concentration. Nanocarriers facilitate slow and sustained release of drug at the targeted site. They potentiate the bioavailability, circulation time and metabolic stability of the drug. Currently various biodegradable nano carriers are available and could be used for drug delivery. Dendrimer are a new class of molecule which can be used for improved drug delivery at nano-scale. Dendrimers are appealing choice for drug delivery due to its unique supra-molecular and interfacial characteristic and it has ability to increase solubility, permeability of drug molecules, high drug loading capacity, good biodegradability and chemically adjustable peripheral functional groups also helps in design of controlled release

formulations (Patri, Majoros *et al.* 2002). Dendrimers based targeted delivery is most emerging, particularly in the delivery of drugs and bio-macromolecules such DNA and siRNA for improving the index of therapeutic potential (Zhu, Liu *et al.* 2019). High level of controlled design and construction of dendrimer in their shape, size, length of branch and their exterior surface monolayer functions distinguished by virtue of these specific features offer as most advantageous carriers in various biomedical and industrial applications (Abbasi, Aval *et al.* 2014). Dendrimers have an excellent capability to improve the solubility, bioavailability and permeability of drugs or molecules for optimization and can be used as nanocarrier in pharmaceutical applications (Santos, Veiga *et al.* 2019). Modern advances in dendrimer chemistry may afford an opportunity to simplify and optimizing the synthesis of dendrimers for various applications with low cost and targeted delivery. In the present study we chemically conjugated the GA with 4.0 G PAMAM dendrimers to extend the bioavailability and explored the molecular mechanism of inhibition of cell proliferation and induction of cell death in HCT-116 cells. In addition effect of PAMAM-GA conjugate was also examined in vivo DL lymphoma induced xenograft mouse mode and drug resistance phenotype developed in HCT-116 cells.

2. Hypothesis

Since past decades extensive research has been progress to explore molecular mechanism and therapeutic targets in area of cancer biology, but still seeking an attention to make progress. In clinical practices chemotherapy is prime therapy has been used against different types of cancer, but after some time it exerts systemic toxicity, adverse side effects and drug resistance. Conventional chemotherapeutic agents are distributed non-specifically in the body where they affect both cancerous and normal cells, thereby limiting the dose achievable within the tumor and also resulting in suboptimal treatment due to excessive toxicities. Similar to other natural polyphenolic derivatives, Gallic acids also show poor bioavailability, limited tissue distribution and rapid degradation, which limit its clinical application to consider as drug candidate. Therefore, here we were interested to improve the bioavailability of GA using PAMAM dendrimers as a drug delivery tool. The present study was aimed to extend the bioavailability of GA via its conjugation with 4.0 G PAMAM to examine its bioavailability, anti-cancer activity and molecular mechanism of cell death in *in vitro* and *in vivo* model system of cancer.

6. Conclusions

Cancer is one of the most lives threading disease and leading causes of death worldwide. In clinical practices chemotherapy is the prime therapy for the treatment of cancer. Chemoththerapeutic drug provies beneficial response at initial stage, but also exerts cytotoxic response, adverse side effects and drug resistance. These are major obstacles for successful application of cancer therapy. Cancer treatment is very much dependent on the method of delivery. The effectiveness of a cancer therapy device is measured by its ability to reduce and eliminate tumors without damaging the healthy tissue. Therefore, a distinct capacity to target cancer is essential for successful therapeutic approach. Many natural derivatives have broad spectrum biological and anti-cancer activity, but could not meet for therapeutic applications due to their low bioavailability. Recent advances in nanotechnology have contributed to novel nanoscale targeting approaches that could be new stratagem for drug delivery. In the present study, Gallic acid (GA) was conjugated with the Polyamidoamine (PAMAM) dendrimer for effective drug delivery. Here, we examined its bioavailability, anti-cancer activity and explored the molecular mechanism of programmed cell death signaling in human colon carcinoma cells (HCT-116) and drug resitance phenotype consisting HCT-116/R cells.

The following salient findings were achieved from the present study:

1. **Biophysical characterization revealed successful conjugation of Gallic acid with PAMAM dendrimers.**

   The conjugation was confirmed by UV-Vis spectroscopy, FT-IR spectral analysis, NMR spectroscopy, and particle size analysis. The UV-Vis spectroscopy showed λmax of 4.0 G PAMAM at 300 nm, whereas λ_{max} of PAMAM-GA conjugate was shifted to 290 nm. Next, FT-IR analysis revealed the appearance of the peak of the amide linkage at 1643 cm^{-1} and aromatic linkage (C=C) at 1465 cm^{-1} that denotes the conjugation of 4.0 G PAMAM dendrimer with GA. The NMR spectra also revealed the formation of PAMAM-GA conjugate with significant changes in integral values and shifts of the secondary -CH_2 group that was observed upon conjugation. The appearance of newer peaks of amide linkage [-NHCO-GA]c at 8.53 ppm and

Ar-H at 8.07- 8.10 ppm, confirms the formation of PAMAM-GA conjugate. PAMAM dendrimer and PAMAM-GA conjugate were further subjected to analysis of particle size distribution. The particle size of 4.0 G PAMAM dendrimer and PAMAM-GA conjugate was found ~ 5.2 nm. However, the conjugation of GA with PAMAM dendrimer did not show a major alteration in the particle size. Minor changes in effective diameter were observed. The average effective diameter of PAMAM dendrimer and PAMAM-GA conjugate was obtained as 198.0 nm and 211.7 nm respectively.

2. **PAMAM-GA conjugate exhibits sustained release and cellular uptake of GA from PAMAM-GA conjugate**

We examined the release study of Gallic acid from PAMAM-GA conjugate in vitro at various time points and cellular uptake in vitro. We found that PAMAM-GA conjugate exhibits slow release than the free Gallic acid. In addition, we noticed that PAMAM-GA conjugate successfully internalized inside the cells in an early time points. The outcomes suggest that conjugation of Gallic acid with PAMAM dendrimer shows improved stability and sustained release that may enhance permeable retention effects and cellular uptake in HCT-116 cells.

3. **PAMAM-GA conjugate reduce the toxicity GA and direct its specificity toward cancer cells**

The results indicate that PAMAM-GA is effective in low concentration to cancer cells and less toxic to normal NIH 3T3 cells. Moreover, GA showing inhibition in cell proliferation at low concentration in HCT-116 as compared to NIH 3T3 cells, but the conjugation of GA with PAMAM dendrimers was showing more efficacious and promising response as compared to free GA. Thus, these results demonstrate that conjugation with PAMAM dendrimer reduces the toxicity of GA. Intrestingly; it may suggest that PAMAM-GA is effective in low concentration to cancer cells and less toxic to normal cells.

4. **PAMAM-GA conjugate inhibits cell proliferation and cell survival of cancer cells**

PAMAM-GA conjugate significantly inhibits cell proliferation of HCT-116, HT-29 and MCF-7 cells. Free GA was found to induce excessive cell death at a lower concentration in both HCT-116 and NIH 3T3 cells, whereas PAMAM-

GA conjugate showed significant cell death to cancer origin cells, but not to the normal NIH 3T3 cells. Next, we examined the effect of PAMAM-GA conjugate on cell survival by examination of the clonogenic ability of the cells. The results showed that PAMAM-GA conjugate inhibits colony formation in a dose and time-dependent manner on HCT-116 cells.

5. **PAMAM-GA conjugate inhibits cell migration by downregulation of MMP-9**

   PAMAM-GA conjugate constantly inhibits the migration ability of HCT-116 cells in a dose and time-dependent manner as compared to the control. Cell migration is associated with upregulated expression of matrix metalloproteinases. The result show HCT-116 cells treated with PAMAM-GA conjugate downregulates the expression of MMP-9.

6. **PAMAM-GA conjugate induces cell death via activating the cascades apoptosis signaling**

   The results show that HCT-116 cells treated with different concentrations of PAMAM-GA conjugate showed Annexin V and PI positive cells at 6, 12 and 24 h. The higher number of Annexin V and PI positive stained cells were found at 12 h. We examined the expression of Bcl-2, cytochrome c, procaspase-3 and PARP in HCT-116 cells by western blotting. We observed that PAMAM-GA conjugate treatment alleviates the expression of the anti-apoptotic protein, Bcl-2 as compared to the control and reduces levels of mitochondrial cytochrome c with a concomitant increase of the cytosolic cytochrome c, downregulation of pro-caspase3 and cleave of PARP on shown time points. The formation of the apoptosome complex was confirmed by co-immunoprecipitation assay. PAMAM-GA conjugate augments the interaction of pro-caspase-9, cytochrome c, and Apaf- 1 in a dose-dependent manner, which indicating apoptosome mediated activation of caspase-9 and caspase-3. In consistent, we found an increased activity of caspase-3 activity on PAMAM-GA conjugate treated HCT-116 cells as compared to the control. Further, Loss of mitochondrial membrane potential (MMP) revealed that GA conjugated with PAMAM dendrimer causes changes in mitochondrial membrane potential and executes mitochondrial-dependent apoptosis in HCT-116 cells. Importantly, HCT-116 cells treated with GA exhibits cell death associated with necrosis whereas PAMAM-GA conjugate restricts cell death by necrotic and direct cell death towards apoptosis.

7. **PAMAM-GA conjugate inhibits the activity of NF-κB (p65) and release of Pro-inflammatory cytokines IL-1β and IL-6**

   Nuclear factor kappa B (NF-κB) is a transcription factor that regulates expression of different sets of genes including proliferation, anti-apoptotic and inflammatory response for cell survival. PAMAM-GA conjugate efficiently inhibits NF-κB activity by restricting the cytosolic to nuclear translocation of p65 in HCT-116 cells. Activation of NF-kB activates secretion of Pro-inflammatory cytokines that is associated with the progression of malignancy. Our results show that PAMAM-GA conjugate inhibits the secretion of pro-inflammatory IL-1β and IL-6. In addition, expression of pro-inflammatory cytokines at transcriptional level was examined by real time PCR. The results show that PAMAM-GA conjugate treated cells downregulates the gene expression of IL-1β and IL-6 mRNA in a dose-dependent manner as compared the control.

8. **MDR-1 over expressed HCT-116 cells show resistant toward treatment of 5-FU**

   Inhibition in cell proliferation with 5-FU drug was examined by MTT assay. Different concentrations ranging from 10 to 600 µg/ml on HCT-116 and HCT-116/R cells exposed to 5-FU for 24h. The HCT-116/R cells show resitant to 5FU tretament as compared to HCT-116 cells, which confirms that MDR-1 overexpression exhibits drug resitance phenotype in HCT-116 cells.

9. **PAMAM-GA Conjugates inhibits cell proliferation of MDR-1 over expressed HCT-116 cells**

   Inhibition of cell proliferation with PAMAM-Gallic acid conjugate was examined by MTT assay with different concentration ranging from 2.5mM, 5mM and 10mM on HCT-116/R cells for 24h. PAMAM-GA treated HCT-116/R cells show inhibition in cell proliferation is dose dependent manner. The results indicate that PAMAM-Gallic acid conjugate significantly inhibits cell proliferation of HCT-116/R cells.

10. **PAMAM-GA Conjugates augments apoptotic cell death in MDR-1 over expressed HCT-116 cells**

Annexin V/PI staining was used to confirm apoptotic cell death in HCT-116/R cells. HCT-116/R cells were treated with different concentration of PAMAM-GA conjugate for 24 h. The result shows a marked increase in nuclear fragmentation along with loss of cellular morphology and massive cell death at 24 h. Further, mitochondrial membrane potential was examined using a mitochondria specific lipophilic fluorescent probe JC-1. In apoptotic cells, due to loss of mitochondrial membrane potential, the dye retains in the cytoplasm and fluoresces green, while in healthy cells, the dye aggregates in the mitochondria and fluoresces red. The results show that PAMAM-GA conjugate potentiate loss of mitochondrial membrane in HCT-116/R cells.

11. **PAMAM-GA Conjugates inhibits accumulation of Rhodaminne 123 MDR-1 over expressed HCT-116 cells**

    MDR1 protein is upregulated in several cancers and responsible for decreased drug accumulation in MDR cells, thereby promoting development of resistance to anti-cancer drugs. Intracellular accumulation of rhodamine-123 (Rh-123) was measured in HCT-116/R cells treated with various concentrations of conjugate (2.5 mM, 5.0 mM, 10.0 mM) for 24 h. PAMAM-GA conjugate-treated HCT-116/R cells showed Rh-123 accumulation as compared to the control cells. Further, we examined P-gp protein expression by western blot and it showed similar result where P-gp was downregulated in dose dependent manner.

12. **PAMAM-GA conjugate reduces tumor volume in Dalton's lymphoma xenograft mouse model**

    The effects of PAMAM-GA conjugate on skin xenograft of Dalton's lymphoma solid tumor bearing mouse was examined. The solid tumor developed mice treated with the Dendrimer, Gallic acid and different concentration of PAMAM-GA conjugate show concurrent reduction in tumor volume as compared to the untreated control group of mice.

13. **PAMAM-GA conjugate reduces lactate dehydrogenase activity and hemolytic toxicity**

    The effect of PAMAM-GA conjugate on lactate dehydrogenase activity was examined from the serum. The results showed that GA and PAMAM-GA conjugate treated mice show reduced LDH activity as compared to the

control group. The result reveals that PAMAM-GA conjugate down regulates LDH activity in tumor-bearing mice, which indicate that it restricts anaerobic metabolism. For the hemolytic toxicity blood collected from healthy untreated mice and treated with different concentration of dendrimer and PAMAM-GA conjugate. The results showed that conjugate has lowered hemolytic toxicity compared to the dendrimer.

14. PAMAM-GA conjugate treatment exhibits improved histopthaological analysis and upregulates the expression of p53 protein

We examined the cellular architecture of the normal, tumor and GA and PAMAM-GA conjugate treated tissues by H & E staining. We observed that untreated normal mouse showed normal muscle tissue architecture. The disease control tumor-bearing mouse showed unhealthy architecture of muscle tissue, increased number of dividing cells and infiltration of lymphocytes. PAMAM-GA conjugate treatment (250µg/ml) showed disappearance of tumor and appearance of apoptotic cells as compared to the control mouse. we examined the effect of PAMAM-GA conjugate on the expression of p53 protein by immunohistochemistry. The results revealed that GA and PAMAM-GA conjugate 250µg/ml (50 mg/kg GA) treated tumor mice showing accumulated expression of p53 as compared to the disease control mouse. Thus results show that PAMAM-GA conjugate can inhabits cell proliferation in tumor bearing mouse.